The plastome-encoded PsaJ subunit is required for efficient Photosystem I excitation, but not for plastocyanin oxidation in tobacco

Mark A. SCHÖTTLER¹, Claudia FLÜGEL, Wolfram THIELE, Sandra STEGEMANN and Ralph BOCK

Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany

The functions of several small subunits of the large photosynthetic multiprotein complex PSI (Photosystem I) are not yet understood. To elucidate the function of the small plastome-encoded PsaJ subunit, we have produced knockout mutants by chloroplast transformation in tobacco (*Nicotiana tabacum*). PsaJ binds two chlorophyll-*a* molecules and is localized at the periphery of PSI, close to both the Lhca2- and Lhca3-docking sites and the plastocyanin-binding site. Tobacco *psaJ*-knockout lines do not display a visible phenotype. Despite a 25 % reduction in the content of redox-active PSI, neither growth rate nor assimilation capacity are altered in the mutants. *In vivo*, redox equilibration of plastocyanin and PSI is as efficient as in the wild-type, indicating that PsaJ is not required for fast plastocyanin oxidation. However,

PsaJ is involved in PSI excitation: altered 77 K chlorophyll-*a* fluorescence emission spectra and reduced accumulation of Lhca3 indicate that antenna binding and exciton transfer to the PSI reaction centre are impaired in Δ *psaJ* mutants. Under limiting light intensities, growth of Δ *psaJ* plants is retarded and the electron-transport chain is far more reduced than in the wild-type, indicating that PSI excitation might limit electron flux at sub-saturating light intensities. In addition to defining *in vivo* functions of PsaJ, our data may also have implications for the interpretation of the crystal structure of PSI.

Key words: Lhca, photosynthesis, Photosystem I, PsaJ, plastocyanin.

INTRODUCTION

PSI (Photosystem I) is a multisubunit protein complex in the photosynthetic membranes of higher plants, algae and cyanobacteria. It consists of 15 core subunits and five different antenna proteins in higher plants [1,2]. PSI catalyses the final step of linear electron transport, the light-driven oxidation of PC (plastocyanin) and the reduction of ferredoxin. Despite the existence of a crystal structure of higher plant PSI [3], the exact functions of several subunits are still unknown. Best understood is the role of the three plastome-encoded subunits, PsaA, PsaB and PsaC, which are essential for PSI assembly and function, because they bind all redox-active cofactors: PsaA and PsaB form the PSI reaction centre heterodimer and bind the majority of the cofactors and antenna chlorophylls (chls), whereas PsaC binds the cofactors F_A and F_B on the PSI acceptor side. In the absence of any of these subunits, functional PSI cannot accumulate in photosynthetic eukaryotes [4,5].

In higher plants, the function of the nuclear-encoded subunits has been elucidated in recent years using RNAi (RNA interference), antisense techniques and insertional mutagenesis in *Arabidopsis thaliana* (reviewed in [6,7]). This work has revealed non-essential functions of most nuclear-encoded PSI subunits, with the exception of PsaD, which is essential for PSI accumulation and is involved in the formation of the ferredoxin-binding site [8,9]. All other knockout mutants displayed much weaker phenotypes, often including a reduced PSI content due to destabilization of the complex and sometimes showing altered antenna binding and exciton transfer to the reaction centre [10–12].

In contrast, the functions of the plastome-encoded small PSI subunits, PsaI and PsaJ, has not yet been resolved in higher plants.

In the unicellular green alga *Chlamydomonas reinhardtii*, PsaJ is required for the stabilization of the PC-binding site [13]. In the absence of PsaJ, a large fraction of photo-oxidized P_{700} (chl-*a* dimer of the PSI reaction centre) is not efficiently reduced by PC or cyt (cytochrome) c_6 , although the PsaF subunit, which forms the actual binding site for both mobile redox carriers, is still present. This has suggested a role of PsaJ in adjusting the conformation of the PC-binding site. These physiological data are circumstantially supported by structural data [3] and cross-linking studies [14] revealing a localization of the J-subunit adjacent to PsaF. Furthermore, in a *Synechocystis* Δ *psaJ* mutant, PsaF and PC binding were also affected [15]. In addition, as PsaJ is known to bind two chl-*a* molecules in higher plants [16] and is also localized close to the binding sites of the Lhca2 and Lhca3 antenna proteins, an involvement in exciton transfer to the PSI reaction centre has been proposed [16]. However, this hypothesis has not yet been addressed experimentally in any photosynthetic eukaryote.

To investigate both the possible role of PsaJ in the interaction between PC and PSI and the proposed function in light harvesting, we have constructed a *psaJ*-knockout mutant in tobacco (*Nicotiana tabacum* L.). The availability of a workable chloroplast transformation technology for tobacco plants facilitates the construction of knockout mutants for plastome-encoded genes and open reading frames [17,18]. The WT (wild-type) copy of the gene of interest is replaced by homologous recombination with a knockout allele generated by gene disruption with a selectable marker, most often the *aadA* gene conferring resistance to the aminoglycoside antibiotics spectinomycin and streptomycin [19,20]. Analysing a *psaJ*-knockout transformant, we demonstrate in the present study that the PsaJ protein is not required for efficient PC oxidation under physiological conditions,

Abbreviations used: chl, chlorophyll; cyt, cytochrome; cyt-bf, cytochrome- b_6/f complex; DDM, β -dodecylmaltoside; EP, electron pair; HP, high-potential form; LHC, light-harvesting complex; LP, low-potential form; P_{700} , chl-*a* dimer of the Photosystem I reaction centre; PC, plastocyanin; PQ, plastoquinone; PS, Photosystem; RFLP, restriction fragment length polymorphism; RMOP, regeneration medium of plants; RNAi, RNA interference; TMPD, tetramethyl-1,4-phenylenediamine; WT, wild-type.

¹ To whom correspondence should be addressed (email schoettler@mpimp-golm.mpg.de).

but that it is instead involved in exciton transfer to the PSI reaction centre.

EXPERIMENTAL

Plant growth

WT tobacco (*Nicotiana tabacum* L. var. Petit Havana) and the $\Delta psal$ transformants were grown on soil in controlled environment chambers at approx. $600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity at the uppermost leaves. The day length was set to 16 h, day temperature was 22°C , and relative humidity was 75 %. Night temperature was 18°C , and relative humidity was decreased to 70 %. For low-light experiments, the growth conditions were as stated above, except that the light intensity was reduced to $20 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

Vector construction and chloroplast transformation

Plastid transformation vector p $\Delta psal$ was derived from a cloned tobacco pDNA fragment. A 1871 bp *SpeI* restriction fragment was subcloned into the unique *SpeI* site of vector pBluescript SK+ which had been dephosphorylated after linearization by treatment with calf intestinal phosphatase. The *psal* reading frame was disrupted by partial digestion with *BglIII* and recovery of the linearized fragment followed by blunting of the overhanging ends by a fill-in reaction with the Klenow fragment of DNA polymerase I from *Escherichia coli* (New England Biolabs). Subsequently, a chimaeric *aadA* gene [21] was inserted as a blunt-end *Ecl136II/DraI* fragment into the blunted *BglIII* site within the *psal* coding region. A clone was selected that contained the plastid selectable marker gene *aadA* in the same transcriptional orientation as the *psal* gene. This clone was designated p $\Delta psal$ and used as a chloroplast transformation vector.

Young leaves from sterile tobacco plants were bombarded with plasmid p $\Delta psal$ -coated $0.6 \mu\text{m}$ gold particles using a biolistic gun (PDS1000He; Bio-Rad). Primary spectinomycin-resistant lines were selected on RMOP (regeneration medium of plants) containing 500 mg/l spectinomycin [21]. Spontaneous spectinomycin-resistant plants were eliminated by double selection on medium containing spectinomycin and streptomycin (each 500 mg/l [21,22]). Several independent transplastomic lines were subjected to three additional rounds of regeneration on RMOP/spectinomycin to enrich the transplastome and isolate homoplasmic *psal*-knockout lines.

Isolation of nucleic acids, PCRs and DNA gel blot analyses

Total plant nucleic acids were isolated from leaf tissue samples by a CTAB (cetyltrimethylammoniumbromide)-based method. For gel blot experiments, DNA samples were digested with restriction enzymes, separated on 0.8 % agarose gels and blotted on to Hybond N nylon membranes (Amersham Biosciences). For hybridization, [α - ^{32}P]dATP-labelled probes were generated by random priming (Multiprime DNA labelling kit, Amersham Biosciences). A PCR product covering the entire *psal* coding region (generated with primers PpsaJf 5'-GGTTTTCATATGCGAGATCTA-3' and PpsaJr 5'-CATGACAATAACTAGAATGAA-3') was used as a probe for the RFLP (restriction fragment length polymorphism) analyses. Hybridizations were carried out at 65°C in Rapid Hybridization Buffer (Amersham Biosciences) following the manufacturer's protocol. DNA samples were amplified with *psal*-specific primers in an Eppendorf thermocycler using Taq DNA polymerase (Promega) and standard protocols (50 ng of total genomic DNA in 50 μl reaction volumes). The PCR programme was 35 cycles of 45 s at 94°C , 90 s at 56°C and 90 s at 72°C with a 4 min extension of

the first cycle at 94°C and a 6 min final extension at 72°C . PCR products were analysed by electrophoretic separation in 1.5 % agarose gels.

Gas-exchange measurements

Leaf assimilation capacity was determined in saturating light [$800 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, generated by a tungsten halogen lamp (Schott)] in a closed cuvette system with a Clark-type oxygen electrode (LD-2, Hansatech). Leaf discs (10 cm^2 surface area) were measured in a 10 % CO_2 -enriched atmosphere, to completely suppress photorespiration. Assimilation was measured until reaching steady state. The chl content of the leaf discs was determined after extraction in 80 % acetone [22]. Assimilation rates were corrected for dark respiration, assuming comparable respiration rates in the light as in darkness [23]. It was shown previously that the assimilation capacity determined under these conditions correlates closely with the maximum capacity of linear electron flux [24].

Thylakoid membrane isolation and treatments with chaotropic agents

Thylakoid membranes were isolated as described [24]. The chl contents and a/b ratio were determined in 80 % acetone [22]. For the *in vitro* stability measurements of PSI, thylakoid membranes equivalent to 200 μg of chl/ml were incubated in medium A (5 mM MgCl_2 , 30 mM KCl and 40 mM Hepes, pH 7.6) as a control or in medium A containing 1 M NaCl, 2 M KI or 2 M NaBr. After 10 min of incubation in the chaotropic salt solution, thylakoids were sedimented by centrifugation at 10000 *g* for 1 min and resuspended in medium A.

Fluorimetry

The 77 K chl-a fluorescence emission spectra were measured using a Jasco F6500 fluorimeter with a red-sensitive photomultiplier. Samples equivalent to 10 μg of chl/ml were excited at 430 nm wavelength (10 nm bandwidth), and emission spectra were measured from 660 to 800 nm wavelength with 0.5 nm steps and a bandwidth of 1 nm. The spectra were corrected for the instrumental response of the photomultiplier.

Room temperature (20°C) chl-a fluorescence of leaves was measured using a Dual-PAM instrument (Walz). The redox state of Q_A and of the PQ (plastoquinone) pool was calculated from $(1 - q_L)$, a parameter more accurate than the commonly used $(1 - q_P)$ [25]. To determine the capacity for state transitions, leaves were excited using a red LED (light-emitting diode) ($\lambda = 660 \text{ nm}$, $80 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity) and either blue light for predominant PSII excitation and PQ pool reduction ($\lambda = 440 \text{ nm}$, $25 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) or far-red light ($\lambda = 715 \text{ nm}$) for preferential excitation of PSI and PQ pool oxidation. Measurements were performed and analysed as outlined in [26].

Cyt-bf (cytochrome b_6f complex) and PSII quantification

The cytochromes of the thylakoid membrane were determined in isolated thylakoids equivalent to 50 μg of chl/ml after destacking in a low-salt medium, to improve the optical properties of the sample [27]. The cytochromes were oxidized by addition of 1 mM ferricyanide and subsequently reduced by addition of 10 mM ascorbate and dithionite, resulting in reduction of cyt f and the high-potential form (HP) of cyt b_{559} (ascorbate – ferricyanide difference absorption spectrum) and reduction of cyt b_6 and the low-potential form (LP) of cyt b_{559} (dithionite – ascorbate) respectively. At each redox potential, absorption spectra were recorded between 575 and 540 nm wavelengths with a spatial resolution

Figure 1 Generation of *psaJ*-knockout plants

(A) Construction of a *psaJ*-null allele by disruption with the *aadA* selection marker. Physical maps of the *psaJ* region in the WT plastid genome and the plastome in Δ *psaJ* plants are depicted. Genes above the line are transcribed from left to right, genes below the line are transcribed in the opposite direction. Relevant restriction sites used for cloning and RFLP analysis are indicated. Sites lost because of ligation to heterologous ends are shown in parentheses. (B) RFLP analysis to confirm homoplasmy and *psaJ* disruption by the chimaeric *aadA* cassette. DNA samples were digested with the restriction enzyme *SacI* which produces a 5.5 kb fragment for the *psaJ* region in the WT (see A). Disruption of the *psaJ* gene with the *aadA* selection marker results in a size increase of the probed restriction fragment by 1.1 kb. As expected, Southern blot analysis (probed with a radiolabelled PCR product covering the entire *psaJ* coding region) confirms the absence of the smaller 5.5 kb WT fragment from the transplastomic lines demonstrating that they are homoplasmic. (C) Phenotypic comparison of WT and Δ *psaJ* plants under standard growth conditions. The transplastomic knockout lines do not show any visibly distinct phenotype when grown in a controlled environment chamber under a light intensity of $600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

of 0.2 nm using a Jasco V550 spectrophotometer with a head-on photomultiplier. The monochromator slit width was set to 1 nm. Difference absorption spectra were deconvoluted, using reference spectra and difference absorption coefficients for the cytochromes as described [27]. PSII contents were calculated from the sum of the cyt *b*₅₅₉ HP and LP difference absorption signals [28].

PC and P₇₀₀ redox kinetics and quantification

Difference absorption signals of PC and P₇₀₀ were measured in the far-red range of the spectrum, essentially as described [29]. The contributions of PC and P₇₀₀ were deconvoluted by measuring difference absorption changes at 830–870 nm (predominantly arising from P₇₀₀) and 870–950 nm (predominantly arising from PC). Measurements were carried out using a novel instrument (Dual-PAM-S) developed by M.A.S. in collaboration with C. Klughammer and U. Schreiber (Walz GmbH, Effeltrich, Germany). This instrument allows the simultaneous determination of both difference absorption signals. Measurements were done on pre-illuminated intact leaves with a fully activated Calvin cycle, so that a limitation of P₇₀₀ photo-oxidation by metabolic NADP⁺ regeneration could be excluded. P₇₀₀ and PC were photo-oxidized using far-red light (715 nm wavelength), which selectively excites PSI. After 10 s of exposure to far-red light, a saturating pulse of red light ($6000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 200 ms duration) was applied and the far-red light was switched off, so that PC and P₇₀₀ could become fully reduced again after the end of the actinic light pulse. The reduction kinetics were fitted with an exponential function to determine the half-times of PC and P₇₀₀ reduction.

For PSI quantification in isolated thylakoids, membranes equivalent to 50 μg of chl/ml were solubilized in medium A (see above) containing 0.2% (w/v) DDM (β -dodecylmaltoside),

100 μM Methyl Viologen as the artificial electron acceptor and 10 mM ascorbate as the electron donor. P₇₀₀, determined as described for measurements on intact leaves, was photo-oxidized by application of a saturating pulse of red light ($6000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity, 200 ms duration). For *in vitro* measurements of P₇₀₀ and PC redox kinetics, 1 μM PC, isolated as described in [29], together with 10 mM ascorbate and 100 μM TMPD (tetramethyl-1,4-phenylenediamine), was used as a donor system.

Protein electrophoresis and immunoblotting

Thylakoid proteins were separated by SDS/PAGE (12% gels) using a Perfect Blue twin gel system (PeqLab GmbH). Proteins were transferred on to a PVDF membrane (Hybond P, Amersham Biosciences) using a semi-dry blot system (SEDEC-M, PeqLab) and a standard transfer buffer (25 mM Tris/HCl and 192 mM glycine, pH 8.3). Immunoblot detection was carried out using the ECL[®] (enhanced chemiluminescence) system (Amersham Biosciences) according to the instructions of the manufacturer. Antibodies against Lhca1–4 and against Lhcb proteins were purchased from Agrisera AB. All immunoblot data were confirmed for three independently grown batches of WT and mutant plants.

RESULTS

Construction of *psaJ*-knockout mutants and isolation of homoplasmic lines

To facilitate the functional analysis of *psaJ* in higher plants, we constructed a *psaJ* knockout by disrupting the reading frame with the selectable marker gene *aadA* (Figure 1A). The *psaJ*-knockout allele was then introduced into the tobacco plastid genome by biolistic chloroplast transformation. Two homoplasmic

recombination events in the regions flanking the disrupted *psaJ* incorporate the knockout allele into the plastid genome where it replaces the functional WT allele (Figure 1A). Selection of bombarded leaf samples for resistance to spectinomycin conferred by the chimaeric *aadA* marker gene yielded several transplastomic lines, of which four were subjected to additional rounds of regeneration and selection to obtain homoplasmic tissue [21]. After three such rounds, plants were regenerated, rooted in sterile culture, subsequently transferred to soil and grown to maturity in the greenhouse. Seeds were obtained and analysis of the T1 generation by seed assays confirmed that the progeny was uniformly resistant to spectinomycin (results not shown). This suggested that the transformed chloroplast genome carrying the *psaJ*-disrupting *aadA* gene was present in a homoplasmic state and was inherited uniparentally maternally, as typical of a chloroplast-encoded trait [30]. All plants from all independently generated transplastomic lines were phenotypically identical (see below) and two independent lines (Nt- Δ *psaJ*#9 and Nt- Δ *psaJ*#20) were selected for further analysis. To verify homoplasmy molecularly and to confirm correct integration of the selectable marker gene *aadA* into the *psaJ* reading frame, RFLP assays (Figure 1B) were performed. These experiments ultimately verified that the WT allele had been replaced by the knockout allele in all copies of the chloroplast genome (Figure 1B, and results not shown). As all photosynthetic parameters (see below) of these two lines were highly similar, only average values for both lines are presented.

Growth phenotype and functional organization of the photosynthetic apparatus in Δ *psaJ* plants

When grown under standard conditions for tobacco plants ($600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 16 h day length), Δ *psaJ* plants did not display any visible phenotype (Figure 1C). Growth, leaf size, number of leaves and flowering time were identical with those of WT plants (Figure 1C). As some phenotypes of photosynthesis mutants become more pronounced with increasing leaf age, we determined all photosynthetic parameters for leaves of different ages from fully grown plants at the onset of flowering. As the youngest leaf, leaf number 4 (from the top of the plant) was analysed, which was already largely expanded. In addition, leaf numbers 6, 9 and 11 were measured, with leaf number 11 being the oldest leaf not yet displaying any visible symptoms of senescence, although assimilation capacity (Figure 2A) and chl content (Figure 2B) were already severely reduced. In both WT and Δ *psaJ* plants, chl contents were highest in the youngest leaves ($525 \text{ mg of chl/m}^2$), and decreased continuously with increasing leaf age to leaf number 11, which contained only approx. 65 % of the chl content of leaf number 4. No significant differences between the WT and Δ *psaJ* plants were observed (Figure 2B). However, interestingly, chl-a/b ratios differed between WT and Δ *psaJ* plants (Figure 2C): in Δ *psaJ* plants, the chl-a/b ratio was slightly increased, the difference from that of the WT plant becoming more pronounced with increasing leaf age. Although such changes of the chl-a/b ratio usually indicate alterations in the stoichiometries of the photosynthetic complexes, leaf assimilation capacities did not differ between WT and mutant plants (Figure 2A): again, the highest assimilation rates were obtained for the youngest leaves [approx. $600 \mu\text{mol of EPs (electron pairs)/mg of chl per h}$], and declined to about half of the maximum assimilation capacity in the old leaves ($275 \mu\text{mol of EP/mg of chl per h}$). These data already indicate that loss of *PsaJ* does not impair linear electron flux under saturating light intensities, suggesting that *PsaJ* is not essential for PSI assembly or function. To determine the molecular basis of the altered chl-a/b ratios, we isolated thylakoids

Figure 2 Assimilation capacities and chl contents are unaltered, but the chl-a/b ratio and PS accumulation are altered in Δ *psaJ* plants

The first fully expanded leaf (number 4 from the top) and leaf numbers 6, 9 and 11 were analysed comparatively to determine possible phenotypic alterations arising during leaf ontogenesis. ●, WT; ○, Δ *psaJ* mutant (*PsaJ*). Leaf assimilation capacities of WT and Δ *psaJ* mutants are identical (A). No effect on the chl content per leaf area was observed (B), but the chl-a/b ratio is slightly elevated in the Δ *psaJ* mutant (C), indicating some changes in thylakoid complex stoichiometries. Thylakoids were isolated from the leaves measured to obtain the datasets displayed in (D–G). PS contents were determined from cyt *b*₅₅₉ (PSII) and P₇₀₀ (PSI) difference absorption signals respectively. Cyt-bf quantification was based on difference absorption signals of cyt *f* and cyt *b*₆. PC was measured in the far-red range of the spectrum. In the Δ *psaJ* mutant, PSII contents (D) were increased significantly, probably as a consequence of reduced PSI accumulation (G). No differences between mutant and WT were observed for cyt-bf (E) and PC (F) contents, which both correlate closely with assimilation capacities (A).

from the leaves used for the assimilation measurements. The contents of all redox-active proteins and protein complexes of the thylakoid membrane were determined by difference absorption spectroscopy and immunoblotting. The contents of PSII (Figure 2D), cyt-bf (Figure 2E) and PSI (Figure 2G), derived from difference absorption signals of cyt *b*₅₅₉ (PSII), cyt *b*₆ and cyt *f* (cyt-bf) and P₇₀₀ (PSI), were determined in solubilized thylakoid membrane fragments. The PC contents, relative to P₇₀₀, were determined in intact leaves, and absolute PC contents were calculated based on the P₇₀₀ quantification in solubilized thylakoids (Figure 2F).

Significant differences between WT and Δ *psaJ* plants were obtained for the two PSs: in WT plants, the PSI contents were approx. $2.3 \text{ nmol of PSI/mol of chl}$, independent of leaf age (Figure 2G).

Figure 3 Immunoblot analysis of marker proteins for the four multisubunit complexes of the photosynthetic light reactions

PsbD (D2-protein) is an essential component of the PSII reaction centre. PetA (cyt *f*) is essential for cyt-*bf* accumulation. PsaC is part of the PSI reaction centre and required for biogenesis and stability of the PSI complex. AtpA is an essential component of the chloroplast ATP synthase. Lanes 1–4 of each blot show undiluted thylakoids from WT leaves (leaf number 4) and a dilution series (50, 20 and 10 % of the undiluted sample) to determine the response range of the antibody. Lanes 5–8 of each blot display WT thylakoids isolated from leaf numbers 4 (lane 5), 6 (lane 6), 9 (lane 7) and 11 (lane 8). Lanes 9–12 of each blot show thylakoids isolated from the corresponding mutant leaves. Double signals for PsbD arise from cross-reactions with the homologous D1 protein; cross-reactions of the AtpA antibody are due to hybridization with the slightly smaller AtpB subunit.

In *ΔpsaJ*-knockout plants, the PSI contents were significantly reduced already in leaf number 4 (approx. 80 % of WT contents), and decreased further with increasing leaf age. In the oldest leaves, only approx. 70 % of the PSI contents in the WT plant were detected. Conversely, PSII accumulated to slightly higher amounts in the *ΔpsaJ* mutant than in the WT plant (Figure 2D). Whereas young WT leaves contained approx. 3.0 mmol of PSII/mol of chl, mutant leaves contained 3.7 mmol of PSII/mol of chl. The increase in PSII contents in *ΔpsaJ* may reflect a redistribution of chl in favour of PSII owing to the reduced PSI content, so that the relative proportion of PSII increases. With increasing leaf age and decreasing leaf assimilation capacity, a reduction of PSII contents was observed in both the WT and the mutant plants. For PC and cyt-*bf* contents (Figures 2E and 2F), no significant differences between WT and *ΔpsaJ* plants were detected. Both cyt-*bf* and PC accumulated to the highest amounts in the youngest leaves, and decreased in parallel with assimilation capacity (Figure 2A).

By and large, these spectroscopic quantifications were confirmed by immunoblot analyses using marker subunits of the photosynthetic complexes (Figure 3). To test the dynamic range of the immunobiochemical detection reactions, a dilution series of the WT samples (100, 50, 20 and 10 %) was used. Proteins from WT and *ΔpsaJ* leaf numbers 4, 6, 9 and 11 were analysed semi-quantitatively by direct comparison with the dilution series. As a diagnostic subunit of PSII, the essential D2 protein encoded by *psbD* was used. Owing to high sequence homology with the slightly smaller D1 protein (PsbA), a double band is always observed with our anti-D2 antibody. No obvious differences between the WT and the *ΔpsaJ* mutant plants were observed with this antibody. This is unsurprising, because the spectroscopic measurements provide a much more precise quantification of complex abundance and the slight increase in PSII contents in the mutant (Figure 2D) was far too small to result in a visual quantitative effect in the immunoblot. For cyt-*bf*, the essential subunit cyt *f* (PetA) was used as a diagnostic subunit [31]. Here, the approx. 50 % decrease of cyt-*bf* content with increasing leaf age is clearly visible. For the PsaC protein,

which binds the stromal iron–sulfur clusters of PSI and is essential for PSI accumulation [5], the signals obtained in the mutants are slightly weaker than in the WT plants. However, the 20–30 % lower PSI amounts measured in the mutant plant are at the limit of detectability with immunoblotting techniques and the spectroscopic data provide a much more reliable dataset here. A strong leaf-age-dependent decrease was observed for the marker subunit of the ATP synthase, AtpA, in both the WT and mutant plants. The weak lower band results from a cross-reaction of our antibody with the AtpB subunit, which shares some sequence similarity with AtpA. In essence, within the detection limits of immunoblots (which are only semi-quantitative), the spectroscopic results were confirmed.

PSI stability and function

To determine whether the reduced PSI contents in the *ΔpsaJ* mutants (Figure 2G) can be attributed to a reduced stability of PSI in the absence of its J-subunit, WT and mutant plants were subjected to stress treatments that potentially affect PSI. To assess PSI stability *in vivo*, tobacco plants were subjected to chilling stress. Plants were transferred to 4 °C and 100 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity. These conditions have been described to selectively damage PSI, owing to inhibition of the Mehler–Asada cycle and accumulation of reactive oxygen species on the PSI acceptor side. In *Arabidopsis thaliana* and barley, a few hours of cold stress were sufficient to result in the loss of up to 50 % of PSI [32,33]. Surprisingly, short-term cold stress of up to 24 h had no effect on the amount of redox-active PSI and on assimilation (results not shown). PSI damage was detectable only after several days of chilling stress, but no differences could be observed between the WT and the *ΔpsaJ* mutant (results not shown). Apparently, tobacco is far more tolerant to cold stress than *Arabidopsis thaliana* or barley.

Additional treatments that potentially destabilize PSI were performed *in vitro* using isolated thylakoids, which were subjected to repetitive cycles of freezing and thawing, to heat stress (10 min of incubation at 70 °C) or to incubation in chaotropic salt solutions. Afterwards, thylakoids were partially solubilized by addition of 0.2 % (w/v) DDM to optimize the optical properties of the sample. Ascorbate was used as an electron donor to keep P_{700} reduced in darkness. Methyl Viologen functioned as an electron acceptor during photo-oxidation. Up to 15 cycles of repetitive freezing in liquid nitrogen and thawing at 35 °C had no significant effect on the amplitude of the P_{700} difference absorption signal (Figure 4A). In response to heat stress (10 min of incubation at 70 °C), a 20 % reduction in the maximum amplitude of P_{700} became apparent. However, no difference between the WT and the *ΔpsaJ* mutant plants was observed. Also, 10 min of incubation with different chaotropic reagents did not result in significantly different effects on the maximum oxidizable fraction of P_{700} in the WT and the mutant plants. Both 2 M KI and 2 M NaBr resulted in some reduction of the P_{700} amplitude, with the effect being slightly more pronounced in mutant thylakoids. Owing to high standard deviations, these differences are not statistically significant. Evidently, the J-subunit is not required to stabilize the intracomplex electron transfer from P_{700} to the artificial acceptor Methyl Viologen.

However, interestingly, solubilization of PSI with 0.2 % (w/v) DDM resulted in a clearly delayed P_{700} reduction in the *ΔpsaJ* mutant after the end of the saturating light pulse (results not shown). To analyse this effect in more detail, and to exclude the possibility that different reduction kinetics of WT and mutant PSI might be due to different PC contents still associated with PSI after solubilization, P_{700} redox kinetics of solubilized PSI particles were

Figure 4 Overall stability of P_{700} in response to stress treatments is unaltered in $\Delta psaj$ thylakoids (A), but the PSI donor side is more prone to damage (B)

(A) Thylakoids isolated from young WT and $\Delta psaj$ (PsaJ) leaves were subjected to 15 repetitive cycles of freezing in liquid nitrogen and thawing at 35°C, to heating to 70°C for 10 min, and to 10 min of incubation with chaotropic salts (1 M NaCl, 2 M KI, 2 M NaBr). Afterwards, the maximum amplitude of photo-oxidizable P_{700} was determined by difference absorption spectroscopy. None of the treatments resulted in selective destabilization of the mutant PSI complex. Heating to 70°C resulted in loss of approx. 25% of PSI in both WT and the $\Delta psaj$ mutant; the other treatments had even smaller effects on the P_{700} amplitude. (B) However, *in vitro* P_{700} redox kinetics of detergent-solubilized thylakoids reveal an impaired P_{700} photoreduction in $\Delta psaj$ mutants. The reduced state of P_{700} was normalized to 0, and the fully oxidized state was normalized to 1. Reduced P_{700} was photo-oxidized by a 200 ms light pulse ($6000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). After the end of the light pulse, the reduction kinetics of P_{700} were analysed. As PSI was disconnected from PSII owing to detergent treatment, 1 μM PC and 100 μM TMPD were added as an artificial donor system. Analysis of redox kinetics revealed that the donor side of $\Delta psaj$ mutants is highly vulnerable to thylakoid solubilization, because a fraction of slowly reduced P_{700} , which is inaccessible to PC, became apparent after addition of 0.2% DDM, whereas the PSI donor side function remained unaltered in WT thylakoids.

measured in the presence of 1 μM PC, with 10 mM ascorbate and 100 μM TMPD as electron donors to PC (Figure 4B). In the WT plant, almost 85% of P_{700} was reduced with a half-time of less than 8 ms by PC. Only a small fraction of approx. 15% of PSI displayed an extremely slow reduction (half-time > 500 ms). As the relative proportion of this fraction was independent of PC contents added to the sample, we conclude that this fraction represents PSI with a damaged donor side, which cannot bind PC efficiently anymore. In the $\Delta psaj$ mutant, the slow fraction was much larger, with 50% of PSI being inefficiently reduced (Figure 4B). Therefore, in the $\Delta psaj$ mutant, the PSI donor side seems to be much more prone to damage or suffers from aberrant conformational changes, comparable with the situation in a *Chlamydomonas psaj* mutant, where *in vitro* PSI redox kinetics also revealed a large fraction of P_{700} not being efficiently

Figure 5 Unaltered *in vivo* redox kinetics of PC and P_{700} in $\Delta psaj$ leaves

PC and P_{700} redox kinetics were measured in intact WT and $\Delta psaj$ leaves (leaf number 4 from the top). The reduced states of PC and P_{700} were normalized to 0, and the fully oxidized states of both components were normalized to 1. The 'high-potential chain' comprising cyt *f*, PC and P_{700} was fully oxidized by a strong light pulse (200 ms duration, $6000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ intensity), and the PSII side of the electron transport chain was concomitantly reduced. (A) Reduction kinetics. At zero time, the saturating light pulse was switched off, and dark reduction of PC and P_{700} by electrons from the PQ pool was traced. PC and P_{700} reduction kinetics of WT and $\Delta psaj$ plants are roughly identical, with average P_{700} reduction half-times of 3.5 ms. PC reduction was slower, as expected given its lower redox potential relative to P_{700} . (B) To analyse the interaction of PC and P_{700} quantitatively, the normalized PC (y-axis) and P_{700} (x-axis) reduction kinetics were plotted against each other, and the apparent redox equilibration constants were calculated. For both WT and $\Delta psaj$, identical k_{app} values of approx. 7.5 were obtained.

reduced by PC or cyt *c* [13]. To test whether defects in the PC– P_{700} interaction are also observable *in vivo*, a detailed analysis of the redox equilibration between PC and P_{700} was conducted on intact leaves.

Redox equilibration of PC and P_{700}

To analyse the *in vivo* redox equilibration of PC and P_{700} , intact leaves were pre-illuminated to fully activate the Calvin cycle and avoid an acceptor side limitation of P_{700} . Complete photo-oxidation of the 'high-potential chain' comprising cyt *f*, PC and P_{700} and complete photoreduction of the PQ pool was achieved by applying an oversaturating light pulse ($6000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 200 ms duration). After the flash, both the oversaturating light pulse and the actinic illumination were switched off (zero time in Figure 5A), and the reduction of PC and P_{700} by electrons from the PQ pool was determined. In Figure 5(A), exemplary kinetics of PC and P_{700} reduction measured in young leaves are shown. The fully oxidized states of PC and P_{700} at the end of the light pulse were normalized to 1, and the completely reduced states were normalized to 0. Reduction half-times of P_{700} of approx. 3.5 ms were obtained, without any significant differences between WT and $\Delta psaj$ mutant plants (Figure 5A). With increasing leaf age, the reduction half-time of P_{700} increased in both WT and mutant plants to approx. 10 ms (results not shown), which reflects the approx. 50% reduction in assimilation capacity of older leaves (cf. Figure 2A). In accordance with the higher redox potential of P_{700} , electrons accumulated in the PC pool only when the majority of P_{700} was reduced. No significant differences between the WT and the $\Delta psaj$ mutant plants were observed

Figure 6 PSI excitation in the *psaJ*-knockout mutant is less efficient in low light

(A) The steady-state redox state of the PQ pool was analysed at light intensities between 0 and $700 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. At low light intensities, PQ is considerably more reduced in the ΔpsaJ mutant (PsaJ) than in the WT plants, despite identical maximum capacities of linear electron flux. The more-reduced redox state of the PQ pool under light-limited conditions indicates a lower rate of PQ oxidation (i.e. PSI excitation and photochemistry), than reduction (i.e. PSII excitation and photochemistry). (B) Phenotype of the ΔpsaJ mutant under low-light conditions. To confirm the reduced efficiency of photosynthesis in the mutants under light-limited conditions, WT and mutant plants were grown at $20 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. When grown under these low-light conditions for 2 weeks, the mutants (right-hand plant) show retarded growth and reduced chl content per leaf area compared with the WT control (left-hand plant).

(Figure 5A). Although the PC and P_{700} reduction kinetics are normally limited by PQ re-oxidation at cyt-bf, a severe disturbance of the interaction between PC and P_{700} , as observed in the mutant *in vitro* (Figure 4B), is expected to result in a visible delay of net P_{700} reduction and an accelerated electron accumulation at PC. This was not observed, strongly arguing against a role of PsaJ in PC oxidation in higher plants. To test for subtle changes in the interaction between PC and P_{700} , the reduction kinetics of both components were plotted against each other (Figure 5B), and the apparent redox equilibration constants (k_{app}) were calculated [29]; k_{app} values in the range 5–12 were obtained. On average, both the WT and the ΔpsaJ mutant plants had a k_{app} of 7.5. Thus neither redox kinetics nor redox equilibration between PC and P_{700} are affected by PsaJ under physiological conditions in tobacco.

Antenna analysis

In addition to its close vicinity to the PC-binding site, PsaJ is also part of the interface of PSI with the Lhca antenna proteins and has been hypothesized to be involved in exciton transfer from the antenna to the PSI reaction centre [16]. To address the possible role of PsaJ in exciton transfer from the peripheral antenna to the PSI reaction centre, light saturation curves of linear electron flux were measured. Under low light intensities, the quantum efficiency of PSII was found to be significantly lower in the ΔpsaJ

Figure 7 Immunoblot analysis of marker proteins for the antenna system

Lanes 1–4 of each blot show undiluted WT thylakoids from leaf number 4, and dilutions to 50, 20 and 10% of the undiluted WT sample, to determine the response range of the antibody. Lanes 5–8 of each blot display WT thylakoids isolated from leaf numbers 4 (lane 5), 6 (lane 6), 9 (lane 7) and 11 (lane 8). Lanes 9–12 of each blot show thylakoids isolated from the corresponding mutant leaves. Whereas no significant differences between WT and ΔpsaJ mutant plants were obtained for Lhcb proteins and the majority of Lhca proteins, the accumulation of Lhca3 was clearly reduced in the mutants.

mutant than in the WT plants. Also, the PQ pool was considerably more reduced in the mutant, as concluded from significantly increased (1–qL) values (Figure 6A). With increasing light intensity, this difference between the ΔpsaJ mutant and the WT plants decreased progressively and eventually disappeared above $600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light intensity (Figure 6A). This suggests that exciton transfer to P_{700} is less efficient in the mutant, so that PSI excitation limits linear electron flux and assimilation under low light intensities. Indeed, when plants were grown under low-light conditions (2 weeks at $20 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), ΔpsaJ transformants developed a clearly discernable mutant phenotype. Growth was retarded and the chl content per leaf area was visibly reduced (Figure 6B). When plants were transferred back to standard light intensities ($600 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), these phenotypic differences disappeared.

PSI antenna organization and antenna function of plants grown under standard light conditions was further analysed using Lhc isoform-specific antibodies (Figure 7) and performing 77 K chl-a fluorescence emission measurements (Figure 8). Similar to the quantification of the photosynthetic complexes (Figure 3), thylakoids isolated from leaf numbers 4, 6, 9 and 11 were used for immunoblot analyses of antenna protein accumulation. For the Lhcb proteins, no significant differences between WT and ΔpsaJ plants were observed (Figure 7). In contrast, analysis of Lhca accumulation revealed a specific depletion of Lhca3 in ΔpsaJ plants, whereas the other Lhca isoforms accumulated to approximately the same amounts as in WT thylakoids. Specific changes in the functional organization of the PSI antenna system are supported further by 77 K chl-a fluorescence emission analysis (Figure 8): 77 K fluorescence emission spectra of WT and ΔpsaJ thylakoids were normalized to the PSII emission maximum at 686 nm wavelength, since no differences in PSII fluorescence emission properties were expected to occur in the ΔpsaJ mutants. Indeed, the low-wavelength part of the fluorescence emission spectrum, which arises from PSII and LHCII (light-harvesting complex II) fluorescence, was unaltered in ΔpsaJ thylakoids

Figure 8 Altered PSI chl-a fluorescence emission signals of $\Delta psaJ$ mutants at 77 K

Chl-a fluorescence of isolated thylakoids equivalent to $10 \mu\text{g}$ of chl/ml was excited at 430 nm. Fluorescence emission of PSII at 686 nm wavelength was normalized to 1. WT thylakoids showed a typical 77 K chl-a fluorescence emission spectrum, with a PSI emission maximum at a wavelength of 733 nm. In the $\Delta psaJ$ mutant (PsaJ), the overall fluorescence emission of the PSI antenna system was reduced, in agreement with reduced PSI and Lhca3 contents. Also, the maximum was blue-shifted from 733 to 728 nm, indicating an altered functional PSI antenna organization.

compared with WT thylakoids. However, the far-red emission maximum of the PSI-LHCI system was clearly blue-shifted in the $\Delta psaJ$ mutant. Whereas a typical emission peak at 733 nm was observed for WT thylakoids, in agreement with previously obtained 77 K emission spectra of tobacco [34], the maximum emission of the $\Delta psaJ$ mutant was shifted to 728 nm (Figure 8). This blue-shift of chl-a fluorescence emission was observed in all leaves of the mutant, independent of their ontogenetic state (results not shown). Also, the overall fluorescence emission from the PSI-LHCI system relative to PSII was reduced in the mutant, which is in good agreement with the decreased PSI content and the slight complementary increase in PSII content (see Figure 2).

To test whether the loss of PsaJ also interferes with the capacity of PSI to bind LHCI, state transitions were induced by blue-light-enriched illumination of young leaves to reduce the PQ pool and induce antenna redistribution in favour of PSI (state 2). Subsequently, the PQ pool was reoxidized by far-red-light-enriched illumination inducing a return to state 1. When the amplitudes of state transitions were calculated [26], no significant differences between the WT ($F_T = 0.85 \pm 0.14$) and the $\Delta psaJ$ mutant ($F_T = 0.72 \pm 0.16$) were found, suggesting that PsaJ is not required for LHCI binding to PSI.

DISCUSSION

Today, the overall function of higher plant PSI is quite well understood, mainly due to the availability of a crystal structure from pea [3] and the characterization of knockout or antisense/RNAi mutants lacking specific nuclear-encoded subunits [6,7]. A notable exception is the limited knowledge of the role of the small plastome-encoded I- and J-subunits of PSI, whose functions have not yet been resolved in higher plants. This is because the generation of plants with transgenic chloroplasts is still significantly more challenging than nuclear transformation [19,20]. The PsaJ subunit is particularly interesting, because, based on the available crystal structural data, a dual function has been proposed: in view of its localization in close vicinity to both the LHCI- and the PC-binding sites, involvement in both PSI excitation and donor side reactions has been discussed. Furthermore, the plastid localization

of the *psaJ* gene was considered to indicate an important function of this small subunit, as the other plastome-encoded PSI subunits, PsaA, PsaB and PsaC, have all been proven to be essential for PSI biogenesis in eukaryotes, whereas the vast majority of the nuclear-encoded subunits are not [7]. However, a comparable suggestion has recently been proven wrong for cyt-bf, as deletion of the plastid-encoded PetL subunit does not result in a visible phenotype [35]. Therefore the assumption that deletion of plastid-encoded subunits of photosynthetic complexes should result in stronger phenotypes than would result from deletion of nuclear-encoded subunits is not necessarily correct.

The function of the J-subunit has been investigated previously in *Chlamydomonas*. Here, a role in the conformational stabilization of the PC-binding site has been identified [13]. Surprisingly, although the impaired PC binding by PSI should be expected to result in a growth phenotype, this was not observed [13]. PSI contents in the *Chlamydomonas* $\Delta psaJ$ mutants were normal, and the role of PsaJ in exciton transfer to the PSI reaction centre was not addressed. In view of these unresolved issues and existing evidence that the functions of photosynthetic proteins can vary considerably between *Chlamydomonas* and higher plants [31], we addressed the functions of PsaJ in tobacco.

In the present study, we have successfully generated homo-plasmic knockout transformants by disrupting the *psaJ* reading frame with the *aadA* antibiotic resistance marker. Our finding that, when grown under standard growth conditions, the knockout lines do not display a visible phenotype clearly demonstrates that PsaJ is not essential for PSI accumulation and function. The absence of a discernable phenotype is in agreement with the unaltered chl content per leaf area and the identical assimilation capacities of WT and knockout plants (Figure 2), and is reminiscent of the situation in *Chlamydomonas*. However, in contrast with *Chlamydomonas*, loss of PsaJ resulted in a 20–30% reduction of PSI content in tobacco which was slightly more pronounced in older leaves (Figure 2). More importantly, loss of PsaJ had no influence on *in vivo* PC-P₇₀₀ interactions and redox equilibration in tobacco (Figure 5). Thus PsaJ is obviously not required for the stabilization of the PC-binding site *in vivo*. However, in response to thylakoid solubilization, the reduction of PSI by PC was impaired in the mutant, whereas the same treatment had no effect on the P₇₀₀ reduction kinetics in the WT. This *in vitro* phenotype closely resembles that of *Chlamydomonas* *PsaJ*-knockout mutants, where the fast reduction of PSI by either PC or cyt *c*₆ was almost completely abolished [13]. This may indicate that, also in higher plants, PsaJ can help to stabilize the PC-binding site. However, this aspect of PsaJ function seems to become relevant only under non-physiological conditions *in vitro*. It is noteworthy in this respect, that the PC-P₇₀₀ interaction studies in *Chlamydomonas reinhardtii* were performed using isolated PSI particles, so that, potentially, the observed effects could also have been caused by the *in vitro* conditions. Remarkably, no effect of the *PsaJ* deletion on the overall growth of *Chlamydomonas* was reported [13], which might indicate that the observed strong effect on PC oxidation may occur only *in vitro*, as demonstrated here for tobacco.

Whereas the role of PsaJ for PSI stability and PC oxidation seems to be of only limited importance in higher plants, our data strongly suggest that its predominant function resides in PSI excitation. This conclusion is supported by several lines of evidence. First, the growth of the $\Delta psaJ$ mutant is retarded under low-light conditions, whereas no distinct phenotype is seen at higher light intensities (Figures 1C and 6B). Secondly, the PQ pool is more reduced in low light in the mutant than in the WT plant (Figure 6A). Thirdly, the 77 K chl-a fluorescence emission spectrum of the PSI-LHCI complex is blue-shifted (Figure 8).

Finally, the accumulation of Lhca proteins is altered in the mutant (Figure 7). Together, these results support a scenario in which PsaJ functions in LHCI binding and exciton transfer to the PSI reaction centre, thereby enlarging the functional PSI antenna cross-section. In the absence of PsaJ, PSI excitation seems to be less efficient, either due to uncoupling of LHCI from PSI or as a consequence of impaired exciton transfer from LHCI to the reaction centre, for which the two chl-*a* molecules bound to PsaJ could be required. The reduced efficiency of PSI excitation results in an imbalance between PSI and PSII excitation in low light and a more reduced redox state of the PQ pool at limiting light intensities. Surprisingly, the mutants are not capable of compensating for this imbalance in PS excitation, although their capacity for state transitions was found to be normal. Therefore the PSI-oxidizing capacity seems to limit linear electron flux in low light. Under high-light intensities, both the growth phenotype and the more reduced redox state of the mutant's PQ pool disappear, because now PS excitation is no longer limiting, but instead linear electron flux itself becomes limiting for assimilation and growth.

The exact molecular basis of the altered antenna function in Δ *psaJ* plants is currently difficult to elucidate: crystal structures indicate that PsaJ is most likely to be located in the direct vicinity of Lhca2 [3,16]. However, owing to the high homology between Lhca2 and Lhca3, the attribution to Lhca2 of the protein structure closest to PsaJ is not unequivocal [36]. Interestingly, our immunoblot analyses revealed that Lhca2 contents remain unaltered in the mutant, while Lhca3 levels are significantly reduced in the knockout plants. Also, the 77 K chl-*a* fluorescence emission data may be easier to reconcile with a predominant effect on Lhca3 accumulation and function than with an effect on Lhca2: at least *in vitro*, the Lhca2 chl-*a* fluorescence emission peaks at a wavelength of 701 nm [37,38] and is thus in the part of the fluorescence spectrum which is still very similar to the WT spectrum (Figure 8). Only at wavelengths above 700 nm do clear differences between Δ *psaJ* and WT thylakoids become apparent: difference spectra of WT– Δ *psaJ* mutant revealed a negative peak at 715 nm and a positive peak at 735 nm (results not shown). The predominant contribution to *in vitro* 77 K fluorescence emission in this spectral range arises from Lhca3 and Lhca4 [37,38]. Although *in vitro* emission from the *Arabidopsis* Lhca3 isoform is maximal at 725 nm [37], *in vivo* data suggest that Lhca3 may emit predominantly in the 730 nm range, probably due to interaction effects with the PSI reaction centre and the other Lhca proteins. Significantly, Lhca3 antisense plants revealed a reduced and slightly blue-shifted 77 K fluorescence emission, comparable with the effects observed in our Δ *psaJ* transformants [39]. Most importantly, the difference spectra between the WT and the Lhca3 antisense plants [39] are practically identical with those obtained here for WT and Δ *psaJ* plants. Thus the observed 77 K spectra are in excellent agreement with the selective reduction of Lhca3 revealed by immunoblots. Taken together, these data support the suggestion that the Lhca protein localized in the direct vicinity of PsaJ is not Lhca2, but rather Lhca3 [36].

Interestingly, an effect of PSI subunit deletion on the accumulation of antenna proteins has also been reported for other small subunits of the complex. For example, *psaK* antisense and T-DNA (transfer DNA) insertion mutants in *Arabidopsis* [12,40] display a reduced accumulation of Lhca3. However, in both PsaK mutants and Lhca3 antisense plants [39], loss or reduced accumulation of Lhca3 results in a concomitant reduction in Lhca2 accumulation, which is most readily explained by a close physical interaction between Lhca2 and Lhca3 [41]. Our Δ *psaJ* mutant is unique in that this concomitant reduction in Lhca2 does not occur. The structural basis for this observation remains to be determined.

Another interesting question concerns the molecular cause of the reduced PSI contents in the Δ *psaJ* mutant. In theory, this could be due either to a slightly less efficient assembly of PSI or to an accelerated degradation. The latter effect could result from a subtle general destabilization of PSI subunit interactions in the absence of PsaJ. Alternatively, in the absence of its J-subunit, the PSI complex could be more accessible to proteolytic breakdown, for example, due to increased exposure of protease-susceptible domains at its surface. Our *in vitro* measurements of PSI stability in response to repetitive freezing and thawing, heat stress and treatments with chaotropic reagents (Figure 4A) argue against a general destabilization of protein–protein interactions within the complex in the absence of PsaJ, as none of these treatments resulted in an accelerated loss of redox-active PSI. Although the PC-binding site may be bound slightly less tightly, our redox equilibration measurements indicate that this effect is not relevant under physiological conditions (Figure 5). Therefore a scenario in which PSI without PsaJ is more prone to proteolytic degradation may seem more likely. However, as no PSI-degrading proteolytic activity has been characterized to date [42], it is currently not possible to test for an increased accessibility of PSI to native thylakoid proteases.

Finally, our finding that maximum assimilation rates in Δ *psaJ* are identical with those in the WT, despite a 20 % reduction in PSI accumulation, represents a potentially important observation. It implies that PSI, at least in WT plants, does not contribute to photosynthetic flux control. Instead, flux control is exerted at the level of cyt-*bf* (Figure 2E), in agreement with previous data [43,44]. Potentially, PC could also contribute (Figure 2F), as its contents decrease in parallel with cyt-*bf* content and assimilation capacity [24]. As the PsaJ mutant plants, despite a 20 % reduction of PSI contents, can support the same maximum assimilation rates as the WT, it seems clear that plants possess an overcapacity of PSI. This overcapacity may be even greater than revealed by the Δ *psaJ* mutant, because cyt-*bf* accumulates to far lower levels than PSI (1.5 mmol of cyt-*bf*/mol of chl compared with 2.3 mmol of PSI/mol of chl). The half-time of PQ reoxidation, the rate-limiting reaction at the cyt-*bf*, is in the range 2–3 ms [45], and is therefore several times slower than PC oxidation by P_{700} and electron transfer through the PSI complex to ferredoxin, which overall requires less than 1 ms. From these values, a several-fold potential overcapacity of PSI, relative to linear electron flux, can be expected, and therefore the absence of any effect of the diminished PSI content in Δ *psaJ* plants on overall assimilation is unsurprising. Currently, it is not understood why higher plants accumulate much higher PSI contents than is theoretically necessary to support electron flux. One plausible explanation could be that, under certain environmental conditions, a high number of PSI complexes is beneficial to compensate for the low-affinity PC binding [46] and/or the inefficient unbinding of PC from PSI [46]. However, a rigorous analysis of the physiological functions of the PSI overcapacity will require modulation of PSI levels without altering PSI subunit composition.

We thank Britta Hausmann for plant cultivation and Dr Stefanie Hartmann for help with vector construction. This project was supported by a grant of the Deutsche Forschungsgemeinschaft to M. A. S. and R. B. (SFB 429, project A12) and by the Max Planck Society.

REFERENCES

- 1 Khroutchova, A., Hansson, M., Paakkariinen, V., Vainonen, J. P., Zhang, S., Jensen, P. E., Scheller, H. V., Vener, A. V., Aro, E. M. and Haldup, A. (2005) A previously found thylakoid membrane protein of 14 kDa (TMP14) is a novel subunit of plant photosystem I and is designated PSI-P. *FEBS Lett.* **579**, 4808–4812

- 2 Ganeteg, U., Klimmek, F. and Jansson, S. (2005) Lhca5: an LHC-type protein associated with photosystem I. *Plant Mol. Biol.* **54**, 641–651
- 3 Ben-Shem, A., Frolow, F. and Nelson, N. (2003) The crystal structure of plant photosystem I. *Nature* **426**, 630–635
- 4 Takahashi, Y., Goldschmidt-Clermont, M., Soen, S. Y., Franzen, L. G. and Rochaix, J. D. (1991) Directed chloroplast transformation in *Chlamydomonas reinhardtii*: insertional inactivation of the *psaC* gene encoding the iron sulfur protein destabilises photosystem I. *EMBO J.* **10**, 2033–2040
- 5 Wostrikoff, K., Girard-Bascou, J., Wollman, F. A. and Choquet, Y. (2004) Biogenesis of PSI involves a cascade of translational autoregulation in the chloroplast of *Chlamydomonas*. *EMBO J.* **23**, 2696–2705
- 6 Scheller, H. V., Jensen, P. E., Haldrup, A., Lunde, C. and Knoetzel, J. (2001) Role of subunits in eukaryotic photosystem I. *Biochim. Biophys. Acta* **1507**, 41–60
- 7 Jensen, P. E., Haldrup, A., Rosgaard, L. and Scheller, H. V. (2003) Molecular dissection of photosystem I in higher plants: topology, structure and function. *Physiol. Plant.* **119**, 313–321
- 8 Ihnatowicz, A., Pesaresi, P., Varotto, C., Richly, E., Scheider, A., Jahns, P., Salamini, F. and Leister, D. (2004) Mutants of photosystem I subunit D of *Arabidopsis thaliana*: effects on photosynthesis, photosystem I stability and expression of nuclear genes for chloroplast functions. *Plant J.* **37**, 839–852
- 9 Haldrup, A., Lunde, C. and Scheller, H. V. (2003) *Arabidopsis thaliana* plants lacking the PSI-D subunit of photosystem I suffer severe photoinhibition, have unstable photosystem I complexes, and altered redox homeostasis in the chloroplast stroma. *J. Biol. Chem.* **278**, 33276–33283
- 10 Ihalainen, J. A., Jensen, P. E., Haldrup, A., van Stokkum, I. H. M., van Grondelle, R., Scheller, H. V. and Dekker, J. P. (2002) Pigment organization and energy transfer dynamics in isolated photosystem I (PSI) complexes from *Arabidopsis thaliana* depleted of the PSI-G, PSI-K, PSI-L, or PSI-N subunit. *Biophys. J.* **83**, 2190–2201
- 11 Jensen, P. E., Haldrup, A., Zhang, S. and Scheller, H. V. (2004) The PSI-O subunit of plant photosystem I is involved in balancing the excitation pressure between the two photosystems. *J. Biol. Chem.* **279**, 24212–24217
- 12 Varotto, C., Pesaresi, P., Jahns, P., Lessnick, A., Tizzano, M., Schiavon, F., Salamini, F. and Leister, D. (2002) Single and double knockouts of the genes for photosystem I subunits G, K and H of *Arabidopsis*: effect on photosystem composition, photosynthetic electron flow, and state transitions. *Plant Physiol.* **129**, 616–624
- 13 Fischer, N., Boudreau, E., Hippler, M., Drepper, F., Haehnel, W. and Rochaix, J. D. (1999) A large fraction of Psaf is nonfunctional in photosystem I complexes lacking the Psaj subunit. *Biochemistry* **38**, 5546–5552
- 14 Chitnis, P. R. (1996) Photosystem I. *Plant Physiol.* **111**, 661–669
- 15 Xu, Q., Odom, W. R., Guikema, J. A., Chitnis, V. P. and Chitnis, P. R. (1994) Targeted deletion of Psaj from the cyanobacterium *Synechocystis* SP PCC-6803 indicates structural interactions between the Psaj and Psaf subunits of photosystem-I. *Plant Mol. Biol.* **26**, 291–302
- 16 Ben-Shem, A., Frolow, F. and Nelson, N. (2004) Light harvesting features revealed by the structure of plant photosystem I. *Photosynth. Res.* **81**, 239–250
- 17 Ruf, S., Kössel, H. and Bock, R. (1997) Targeted inactivation of a tobacco intron containing open reading frame reveals a novel chloroplast-encoded photosystem I-related gene. *J. Cell Biol.* **139**, 95–102
- 18 Hager, M., Biehler, K., Illerhaus, J., Ruf, S. and Bock, R. (1999) Targeted inactivation of the smallest plastid-genome encoded open reading frame reveals a novel and essential subunit of the cytochrome-*b₆* complex. *EMBO J.* **18**, 5834–5842
- 19 Maliga, P. (2004) Plastid transformation in higher plants. *Annu. Rev. Plant Biol.* **55**, 289–313
- 20 Bock, R. and Khan, M. S. (2004) Taming plastids for a green future. *Trends Biotechnol.* **22**, 311–318
- 21 Svab, Z. and Maliga, P. (1993) High frequency plastid transformation in tobacco by selection for a chimaeric *aadA* gene. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 913–917
- 22 Porra, R. J., Thompson, W. A. and Kriedemann, P. E. (1989) Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* **975**, 384–394
- 23 Raghavendra, A. S. and Padmasree, K. (2003) Beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. *Trends Plant Sci.* **8**, 546–553
- 24 Schöttler, M. A., Kirchhoff, H. and Weis, E. (2004) The role of plastocyanin in the adjustment of photosynthetic electron transport to the carbon metabolism in tobacco. *Plant Physiol.* **136**, 4265–4274
- 25 Kramer, D. M., Johnson, G., Kiirats, O. and Edwards, G. E. (2004) New fluorescence parameters for the determination of *Q_A* redox state excitation energy fluxes. *Photosynth. Res.* **79**, 209–218
- 26 Lunde, C., Jensen, P. E., Haldrup, A., Knoetzel, J. and Scheller, H. V. (2000) The PSI-H subunit of photosystem I is essential for state transitions in plant photosynthesis. *Nature* **408**, 613–615
- 27 Kirchhoff, H., Mukherjee, U. and Galla, H. J. (2002) Molecular architecture of the thylakoid membrane: lipid diffusion space for plastoquinone. *Biochemistry* **41**, 4872–4882
- 28 Lamkemeyer, P., Laxa, M., Collin, V., Li, W., Finkemeyer, I., Schöttler, M. A., Holtkamp, V., Tognetti, V. B., Issakidis-Bourguet, E., Kandlbinder, A. et al. (2006) Peroxiredoxin Q of *Arabidopsis thaliana* is attached to the thylakoids and functions in context of photosynthesis. *Plant J.* **45**, 968–981
- 29 Kirchhoff, H., Schöttler, M. A., Maurer, J. and Weis, E. (2004) Plastocyanin redox kinetics in spinach chloroplasts: evidence for disequilibrium in the high potential chain. *Biochim. Biophys. Acta* **1559**, 63–72
- 30 Bock, R. (2001) Transgenic plastids in basic research and plant biotechnology. *J. Mol. Biol.* **312**, 425–438
- 31 Monde, R. A., Zito, F., Olive, J., Wollman, F. A. and Stern, D. B. (2000) Post-transcriptional defects of tobacco chloroplast mutants lacking the cytochrome-*bf* complex. *Plant J.* **21**, 61–72
- 32 Zhang, S. and Scheller, H. V. (2004) Photoinhibition of photosystem I at chilling temperature and subsequent recovery in *Arabidopsis thaliana*. *Plant Cell Physiol.* **45**, 1595–1602
- 33 Scheller, H. V. and Haldrup, A. (2005) Photoinhibition of photosystem I. *Planta* **221**, 5–8
- 34 Bondarava, N., De Pascalis, L., Al-Babili, S., Goussias, C., Golecki, J. R., Beyer, P., Bock, R. and Krieger-Liszka, A. (2003) Evidence that cytochrome *b₅₅₉* mediates the oxidation of reduced plastoquinone in the dark. *J. Biol. Chem.* **278**, 13554–13560
- 35 Fiebig, A., Stegemann, S. and Bock, R. (2004) Rapid evolution of RNA editing sites in a small non-essential plastid gene. *Nucleic Acids Res.* **32**, 3615–3622
- 36 Jolley, C., Ben-Shem, A., Nelson, N. and Fromme, P. (2005) Structure of plant photosystem I revealed by theoretical modeling. *J. Biol. Chem.* **280**, 33627–33636
- 37 Castelletti, S., Morosinotto, T., Robert, B., Caffari, S., Bassi, R. and Croce, R. (2003) Recombinant Lhca2 and Lhca3 subunits of the photosystem I antenna system. *Biochemistry* **42**, 4226–4234
- 38 Croce, R., Morosinotto, T., Ihalainen, J. A., Chojnicka, A., Breton, J., Dekker, J. P., van Grondelle, R. and Bassi, R. (2004) Origin of the 701 nm fluorescence emission of the Lhca2 subunit of higher plant photosystem I. *J. Biol. Chem.* **279**, 48543–48549
- 39 Ganeteg, U., Strand, A., Gustafsson, P. and Jansson, S. (2001) The properties of the chlorophyll a/b-binding proteins Lhca2 and Lhca3 studied *in vivo* using antisense inhibition. *Plant Physiol.* **127**, 150–158
- 40 Jensen, P. E., Gilpin, M., Knoetzel, J. and Scheller, H. V. (2000) The PSI-K subunit of photosystem I is involved in the interaction between light-harvesting complex I and the photosystem I reaction center core. *J. Biol. Chem.* **275**, 24701–24708
- 41 Jansson, S., Andersen, B. and Scheller, H. V. (1996) Nearest-neighbor analysis of higher-plant photosystem I holocomplex. *Plant Physiol.* **112**, 409–420
- 42 Adam, Z., Rudella, A. and van Wijk, K. J. (2006) Recent advances in the study of Clp, FtsH and other proteases located in chloroplasts. *Curr. Opin. Plant Biol.* **9**, 234–240
- 43 Anderson, J. M. (1992) Cytochrome-*bf* complex: dynamic molecular organization, function and acclimation. *Photosynth. Res.* **34**, 341–357
- 44 Anderson, J. M., Price, G. D., Chow, W. S., Hope, A. B. and Badger, M. R. (1997) Reduced levels of cytochrome *bf* complex in transgenic tobacco leads to a marked photochemical reduction of the plastoquinone pool, without significant change in acclimation to irradiance. *Photosynth. Res.* **53**, 215–227
- 45 Hope, A. B. (2000) Electron transfers amongst cytochrome *f*, plastocyanin and photosystem I: kinetics and mechanisms. *Biochim. Biophys. Acta* **1456**, 5–26
- 46 Finazzi, G., Sommer, F. and Hippler, M. (2005) Release of oxidised plastocyanin from photosystem I limits electron transfer between photosystem I and cytochrome *b₆f* complex *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 7031–7036

Received 18 October 2006/19 December 2006; accepted 9 January 2007

Published as BJ Immediate Publication 9 January 2007, doi:10.1042/BJ20061573